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Genetics

Base substitutions, frameshifts, and small deletions constitute ionizing radiation-induced point mutations in mammalian cells

(mutational specificity/radiation mutagenesis)

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ABSTRACT **The relative role of point mutations and large genomic rearrangements in ionizing radiation-induced mutagenesis has been an issue of long-standing interest. Recent studies using Southern blotting analysis permit the partitioning of ionizing radiation-induced mutagenesis in mammalian cells into detectable deletions and major genomic rearrangements and into point mutations. The molecular nature of these point mutations has been left unresolved; they may include base substitutions as well as small deletions, insertions, and frameshifts below the level of resolution of Southern blotting analysis. In this investigation, we have characterized a collection of ionizing radiation-induced point mutations at the endogenous adenine phosphoribosyltransferase (*aprt*) locus of Chinese hamster ovary cells at the DNA sequence level. Base substitutions represented ≈2/3 of the point mutations analyzed. Although the collection of mutants is relatively small, every possible type of base substitution event has been recovered. These mutations are well distributed throughout the coding sequence with only one multiple occurrence. Small deletions represented the remainder of characterized mutants; no insertions have been observed. Sequence-directed mechanisms mediated by direct repeats could account for some of the observed deletions, while others appear to be directly attributable to radiation-induced strand breakage.**

Although ionizing radiation was the first known mutagen (1), relatively little is known about the molecular mechanisms of ionizing radiation-induced mutagenesis in mammalian cells. Similarly, despite intensive efforts to determine the mutagenic and carcinogenic risk of low dose exposure (2), this question has also not been satisfactorily resolved. Recent efforts toward both goals have increasingly converged due to the expectation that a more fundamental understanding of the mechanisms of radiation mutagenesis will result in a more rational basis for risk estimation.

One approach has been the use of Southern blotting analysis to investigate the mutational specificity of ionizing radiation in mammalian cells. This technique is primarily useful as a means of assessing the fraction of radiation-induced mutation attributable to major genomic alterations such as large insertions, deletions, or translocations. However, Southern analysis cannot detect deletions or insertions of <50 base pairs (bp) as well as most base substitution and frameshift events. Several recent reports (3–8) vary widely in Southern analysis-derived estimates of the percentage of gross alterations (16–80%) in ionizing radiation-induced mutation of mammalian cells. The range probably reflects differences in the systems used.

Radiation-induced mutational events below the limits of resolution of Southern analysis have only been indirectly accessible.‡ The induction of mutation in genes essential for cell viability provides one approach; only base substitutions and in-frame deletions or insertions are expected to be recovered in this type of marker. Ionizing radiation has a well-documented inability to induce ouabain resistance, a mutation of the essential Na^+,K^+-ATPase gene (9–14). This has been interpreted as an inability of ionizing radiation to induce base substitutions in mammalian cells. However, Liber *et al.* (15) reported x-irradiation to induce mutation at two other essential loci. Thus, the failure of x-rays to induce ouabain resistance may be attributable to a peculiarity of the target sequence rather than an inability to cause base-substitution events.

An analysis of ionizing radiation-induced mutational events below the limits of resolution of Southern analysis requires DNA sequence analysis. Several systems to study specificity at the DNA sequence level in prokaryotes have been reported (16–18). Analyses of sequence specificity at endogenous loci of mammalian cells face logistical difficulties associated with cloning and sequencing large numbers of independent mutant alleles and have, therefore, been limited (19). Shuttle vectors, which are able to be propagated in either bacteria or mammalian cells, have been used in studies of the sequence specificity of mutagenesis (20). The selectable markers carried in these vectors are relatively easy to recover for sequence analysis but are subject to a remarkably high frequency of mutation and rearrangement (20). The utility of these systems as models for endogenous cellular genes therefore remains in question.

We have developed a methodology for the rapid cloning and sequencing of mutant alleles at the endogenous adenine phosphoribosyltransferase (*aprt*) locus of Chinese hamster ovary (CHO) cells. The critical aspects of this approach involve the *in vivo* recombinational rescue of an *aprt*-carrying λ phage from a genomic library prepared from a CHO strain that is hemizygous for *aprt* (an autosomal locus). The small size of the gene [2.6 kilobases (kb)] facilitates cloning and subsequent sequence analysis.

We have recently reported a Southern blotting analysis of a collection of spontaneous and γ-ray-induced $APRT^-$ mutants (4) in which ≈84% of the γ-ray-induced mutants were classified as "point mutations." This class of events would include base substitutions as well as small deletions, insertions, and frameshifts below the level of deletion of Southern blotting analysis. In this report, the sequence alterations of 16 γ-ray-induced point mutations are presented.

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‡It has become the practice to classify mutations that cannot be detected by Southern blotting as point mutations (e.g., see refs. 3–8). In reality, this includes not only base substitutions and frameshifts but also deletions and insertions up to ≈50 bp long.

MATERIALS AND METHODS

Cell Culture Conditions, Mutant Selection, and Irradiation. The maintenance and selection of the wild-type strain and γ-ray-induced mutants have recently been detailed (4). Briefly, the parental cell line used in these experiments was D422, an *aprt* hemizygote derivative described by Bradley and Letovanec (22). Cells were maintained in α minimal essential medium supplemented with 2.5% fetal calf serum and 2.5% heat-inactivated horse serum. APRT$^-$ mutants were selected by seeding 5×10^5 cells per 100-mm Petri dish in medium containing 0.4 mM 8-azaadenine, a toxic adenine analogue.

Each γ-ray-induced mutant was recovered from an independent culture of 4×10^6 cells exposed to 5.0 Gy of ^{137}Cs irradiation at a dose rate of 0.7 Gy/min. The resultant surviving fraction was 0.16; the induced mutation frequency in irradiated cultures was 23×10^{-6}; and the spontaneous mutation frequency was 1.8×10^{-6}.

Cloning and Sequencing Methodologies. These procedures (P.J.J., A.J.G., E.A.D., and B.W.G., unpublished results) are based on methodologies described by Seed in Maniatis *et al.* (23). In brief, genomic DNA is double-digested with *Hin*dIII and *Bgl* II, producing a 4.3-kb restriction fragment carrying the mutant gene. After a size-fractionation step, the DNA is ligated into a defective ($A^-_{am}B^-_{am}$) λ vector. The primary libraries in this double amber cloning vector are amplified on a bacterial host harboring the plasmid pPDJ10, which contains the following essential features: a *supF* gene capable of suppressing the amber mutations of the λ vector; a 0.7-kb fragment of Chinese hamster DNA flanking the 2.6-kb *aprt* gene but contained on the 4.3-kb genomic fragment; a phage M13 origin of replication (M13 ori); and a ColE1 plasmid origin of replication (ColE1 ori). Homologous recombination between the library phage containing the sought after 4.3-kb *aprt* fragment and the plasmid (the recombination frequency is $\approx 10^{-3}$) results in the incorporation of the plasmid and its *supF* gene into the phage genome. These recombinants can be selected by titering the amplified library on a nonsuppressor host.

The entire *aprt* gene can be recovered from the λ vector on a single 5.7-kb *Bam*HI fragment also containing the ColE1 *ori*, the M13 *ori* and the *supF* gene. This fragment is circularized, and used to transform an F′ host strain using the plasmid-encoded *supF* gene as the selectable marker. Single-stranded DNA, suitable for dideoxy sequencing, can be readily obtained by superinfection with wild-type M13 phage (24) producing a mixed population of single-stranded plasmid DNA, and M13 DNA is packaged into M13 coats. The *aprt* allele is then sequenced by the method of Sanger *et al.* (25) using a series of *aprt*-specific primers (P-L Biochemicals).

RESULTS

The class of events defined as "point mutations" in Southern blotting analysis includes a wide variety of mutational events. Table 1 summarizes the point mutations observed, including the type of sequence alteration, the position within the *aprt* sequence, and, where relevant, the resultant amino acid change. With a single exception, all of the base substitutions occur in the coding sequence and result in an amino acid alteration (Table 1). The single exception, X-72, involves an A·T to C·G transversion within the termination codon. As a consequence of this change, a carboxyl-terminal extension of 22 amino acids is predicted. Each mutant was sequenced at least through its entire coding sequence as well as much of the introns and 5′ and 3′ flanking sequences. No alterations of intron sequences or at intron/exon junctions (i.e., splice sites) were observed in this collection.

The types of point mutations recovered are catalogued in Table 2. Base substitutions represented 11/16 or ≈70% of all the mutants analyzed. Five frameshift deletions were also observed and account for the remaining 30% of the total. No insertion events were recovered.

Table 1. Sequence analysis of γ-ray-induced *APRT*$^-$ mutants

Mutant	Sequence alteration	Position	Remarks
X-1	Deletion	224–225	
X-7	TV T → G	77	Phe → Cys
X-9	Deletion	1411–1414	
X-10	TV G → C	1351	Gly → Ala
X-21	Deletion	(1821–1828)–(1837–1843)	
X-31	TV A → T	1902	Gln → Leu
X-32	TV A → T	1902	Gln → Leu
X-33	TS T → C	1631	Val → Ala
X-34	Frameshift	1379	
X-38	TV C → A	273	His → Asn
X-39	TV T → G	1872	Leu → Arg
X-42	TS G → A	1639	Asp → Asn
X-44	TS G → A	210	Asp → Asn
X-45	Deletion	1829–1832	
X-65	TS G → A	1321	Gly → Asp
X-72	TV A → C	1912	Stop → Cys

TV, transversion; TS, transition.

A further analysis of the base substitution events is presented in Table 3. Despite the relatively small number of mutants analyzed, every possible type of base substitution event has been observed. Transversions appear to occur more frequently at A·T base pairs (5/7) as compared to transitions (1/4). In addition to the wide variety of base substitutions recovered, an even distribution throughout the coding sequence of the gene has also been observed. Only one site is represented more than once. This is the A·T to T·A transversion at position 1902 (Table 1). The distribution of mutated sites along the gene is depicted graphically in Fig. 1.

Table 4 presents a listing of the γ-radiation-induced deletion and frameshift mutations. The deleted bases are bracketed and separated from the surrounding sequence by spacing. The size of the deletions ranged from 1 to 18 bp. Interestingly, the deletion breakpoints in X-21 and X-45 may be the same (Table 4). However, due to the presence of direct repeats at their endpoints, the precise position of the breakpoints is uncertain. The frameshift X-45 may be viewed slightly differently, since the affected sequence is actually a run of three repeated 2-bp units (TGTGTG). The direct repeats surrounding the deleted sequence in these mutants suggests the occurrence of a sequence-directed deletion event involving slippage and mispairing (see *Discussion*).

DISCUSSION

Most mutational spectra are characterized by hot spots (26), sites at which mutation is clustered. Hot spots may be the result of a number of factors including damage distribution, dose, repair efficiency, local DNA structure, and the relative importance of a given codon for protein structure and function. The ionizing radiation-induced mutational spectrum is unique in lacking obvious hot spots (Table 1). This contrasts markedly with the spontaneous mutational spec-

Table 2. Classes of point mutation induced by ionizing radiation

Class	Number
Base substitution	11
Deletions*	5
Insertions*	0

*Events listed here are below the level of detection by Southern blotting analysis.

Table 3. Ionizing radiation-induced base substitution

	Number
Transitions	
G·C → A·T	3
A·T → G·C	1
Total	4
Transversions	
T·A → G·C	3
A·T → T·A	2
C·G → G·C	1
G·C → C·G	1
Total	7

trum that we have recently described (27), which is characterized by prominent hot spots. For example, a G·C to A·T transition at position 241 was observed in 7/30 independently recovered mutations. Several other positions were also characterized by multiple independent events.

The results presented in Table 3 emphasize the diversity of base substitutions induced by ionizing radiation. Among a sample of only 11 base substitutions, each of the 6 possible substitution events is represented. In contrast, the spontaneous mutational spectrum (27) is characterized by a predominance of G·C to A·T transitions, which accounted for almost 80% of the total base substitutions recovered. The wide variety of base substitutions induced by ionizing radiation reported here is consistent with the broad specificity of ionizing radiation-induced base substitutions in prokaryotes (28–30) and in lower eukaryotes (31–33).

The distribution of mutated sites within the *aprt* gene was also widely scattered (Fig. 1, Table 1) and reminiscent of the lack of specificity in the types of base substitution induced (Table 2). Only one site of multiple occurrence was observed among base substitutions. Two deletions overlapped and may share a common break point (Table 4). The largely random distribution of mutation is consistent with the hypothesis that ionizing radiation deposits energy into DNA in a more or less random fashion. It also contrasts sharply with that obtained for spontaneous mutation (27).

With one exception, the base substitutions result in a coding sequence alteration (Table 1). Mutant X-72 has occurred within the translation termination codon resulting in the production of a cysteine codon in its place (Table 1) and an extension of the open reading frame to include an additional 22 amino acids. Other base substitutions have occurred in close proximity to the 3′ terminus of the coding sequence including the one site of multiple independent events (position 1902; Table 1). This region may therefore represent an important functional domain of the APRT protein or be important in the three-dimensional folding pattern of the active protein.

An examination of the γ-ray-induced deletions suggests that two types of mechanisms may be operable: sequence-directed events and random breakage. The dependence of frameshifts and deletions on repeated sequences has been well established (34–36). The original model for frameshift mutagenesis (36) invoked structural intermediates involving the misalignment of one copy of a direct repeat sequence on

Table 4. Target sequences for ionizing radiation-induced deletions

Mutant line	Sequence alteration*
X-1	220 230 TCTCGCCCCT[CC]TGAAGGACCC
X-9	1400 1420 AGCCTCCTATG[CTCT]CGAGTATGGC
X-21	1820 1845 GAGGTGGTGGAG[TGTGTGAGCCTGGTGGAG]CTGACCT
X-34	1370 1390 AGCGAGGGAA[G]CTGCCAGGCCC
X-45	1820 1840 GGTGGTGGAG[TGTG]TGAGCCTGGTGGAGC

The events listed here are below the level of detection of Southern blotting analysis.

*The precise position of the breakpoints in those cases involving direct repeats (X-9, X-21, X-45) is ambiguous and could lie anywhere within the repeated sequences (underlined). The breakpoints shown here represent only one possibility. In the cases of X-21 and X-45, the breakpoints shown illustrate that an identical breakpoint could account for the observed deletions in each strain.

the complement of a nearby second copy. The slippage and misalignment results in the deletion of one copy of the repeated unit and all of the intervening sequence between the repeats.

Although the data base is still small, it appears that the endpoints of deletions may not be random. The 18-bp deletion in X-21 is flanked by an 8-bp direct repeat at each end of the deleted sequence (Table 4) and the 4-bp deletion in X-45 is surrounded by a 2-bp direct repeat. Such deletions, flanked by repeats, are predicted by DNA misalignments (34, 36, 37). The precise location of the breakpoint is indeterminate because of the repeated sequences that flank the deletion. However, X-21 and X-45 may share one identical breakpoint (Table 4). A possible explanation for the clustering of γ-ray-induced deletions is that an induced break at the site of a potential misalignment event greatly enhances the opportunities for such a misalignment to occur. Studies in *Escherichia coli* indicate that DNA discontinuities (even those produced during a repair process) can result in frameshift events (21, 38). Three large deletions were detected by us using Southern blotting analysis (4); restriction mapping suggested that both breakpoints for all three deletions are close and possibly identical.

In contrast to the deletions in X-21 and X-45, the deletion events observed in X-1 and X-34 are not readily accounted for as slippage events mediated by nearby repeats. These dele-

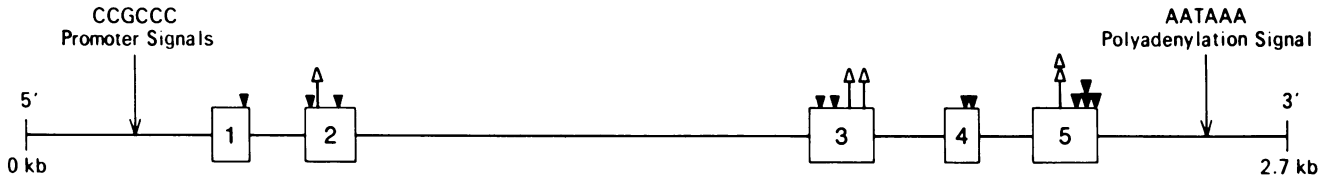

FIG. 1. The distribution of the sites of γ-ray-induced point mutations within the *aprt* gene. The *aprt* gene is drawn to scale with the 5 exons shown as open boxes. Base substitutions are depicted as solid inverted triangles, and deletions and frameshifts are open upright triangles. Sites at which two independent events have been recovered are indicated by stacked symbols.

tions may be the result of DNA strand breakage caused by γ-radiation. These would then differ in origin with mutations mediated by repeated sequences. Events mediated by repeats may be the consequence of attempted DNA repair, while those mutations not readily explained as slippage events may reflect the direct effects of ionizing radiation-induced DNA strand breakage.

In our earlier report (4), we partitioned γ-ray-induced mutants into two classes, major genomic rearrangements (16%) and point mutations (84%). The sequence analysis of 16 of these point mutations enables an extension of the attribution of radiation-induced mutagenesis to specific types of molecular events. Among the point mutations, base substitutions were found to account for 11/16, while small deletions and frameshifts represent 5/16. This permits an estimation of the induction of these events on a per rad basis. Base substitutions represent $11/16 \times 0.84$ or 58% of the mutations at the *aprt* locus. They are induced at a frequency of (23×10^{-6}) (0.58) or $13.3 \times 10^{-6}/5.0$ Gy; on a per rad basis, the frequency is 2.7×10^{-8}. For the frameshift and small deletion events, the corresponding value is 1.4×10^{-8} per rad. While the relative proportions of large genomic alterations vary among different genetic systems, the per rad estimate for the frequency of intragenic events may be more generalizable.

The striking contrast between the spontaneous and radiation-induced mutational spectra in both the type and distribution of mutations (Fig. 1; ref. 27) has important implications for the estimation of low dose radiation effects. Mutational spectra are predicted to be sensitive indicators of low dose effects. Even a 2-fold increase in mutation frequency would mean that 50% of the recovered mutants were induced, enough to cause a major shift in the mutational spectrum. We therefore feel that the development of mutational spectra can aid in the development of a rational basis for risk estimation and exposure regulation.

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